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TITLE: A Solid Support Synthesis and Novel Conjugation Methods of Breast Tumor Associated Antigen: Toward the Development of Cancer Vaccines

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13. ABSTRACT *(Maximum 200 Words)*

N-linked glycopeptides and glycoproteins are important biomolecules. They been revealed to involve a various biological affairs. For the first time, we have achieved the total synthesis of a high mannose N-linked glycopeptide carrying full H-type II human blood group. In this study, we have developed (a) an efficient method for constructing core high mannose structure which is essential for all N-linked glycoproteins; (b) a chemically selective and stereoselective method for N-glycosylation of peptide, which will be widely used for making N-glycopeptide for biological study; (c) an efficient synthetic method to prepare the suitably protected lactosamine spacer; (d) an efficient method to construct H-type trisaccaride unit.

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Table of Contents

Toward Fully Synthetic Homogeneous Glycoproteins: A High Mannose N-linked Glycopeptide Carrying Full H-Type II Human Blood Group Specificity

Introduction

Carbohydrate domains in the context of glycolipids and glycoproteins may carry significant message.[1] The major breakthroughs in the detection, purification, sequencing and spectroscopic analysis of glycans have enabled a growing appreciation for the role of glycobiology in vital life processes.[2] However, carbohydrate components from natural sources are usually heterogenerous and their purification in large scale is extremely difficult, which has been a major obstacle for glycobiological studies. Therefore, chemical synthesis of glycans continues to be an important approach to providing access to well selected, homogenous but realistically complex probe structures for elucidating the relationship of glycoarchitecture and function.[3]

Generally speaking, glycoproteins are of two major types. O-linked type is typical with terminal linkage of GalNAc to serine or threonine via an α-O-glycosidic linkage. The N-linked glycoproteins are formed through a β-N linkage of an asparagines, to a GluNAc at the reducing end of oligosaccharide.[4]

For the decades, chemical synthesis of N-linked glycopeptides and glycoproteins has been a challenging issue.[5] Most recently, we have made a significant progress in this aspect at the Bioorganic Chemistry Lab, Sloan-Kettering Cancer Institute. Presented here is our study toward the total synthesis of glycopeptide structure **51**.

Body

1. An efficient synthesis of key common high mannose sequence.

(αMan1-6)(αMan1-3)(βMan1-4)(βGluNAc(1-4)βGluNAc(core sequence) is the key sequence in glycoproteins. It essentially exists in all N-linked glycoproteins. From the chemical point of view, it contains many difficult oligosaccharide linkages such as β-mannoside, β1-4 GluNAc linkage. Therefore, an efficient synthesis of the core structure **21** containing those difficult linkage sequences is preliminary to access a complex glycoprotein. We started our synthesis from monosaccharide.

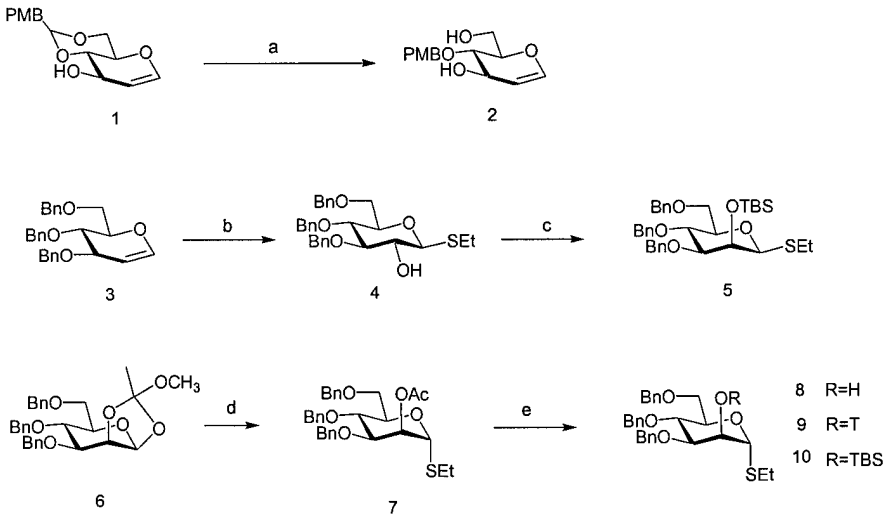


Figure 1, a, DIBAl-H/DCM, 75%; b, 1. DMDO/DCM; 2. EtSH/TFAA; 89% in two steps; c; 1. DMSO/Ac_2O, 2. $NaBH_4$/DCM/MeOH, 76% intwo steps, 3. TBSOTf/DCM/Et_3N, 93%; d, EtSH/$HgBr_2$/CH_3CN, 95%; e, 1. NaOMe/MeOH, 2. TBSOTf/DCM/Et_3N, for 10, 93%, or toloyl chloride/DCM/Et_3N, 75%;

As shown in Figure 1, the benzylidene in compound **1**, prepared by one step selective protection, can be opened selectively by DIBAL-H to afford 3,6-dihydroxylated glycal **2** in 75%. .

Mannosides **5** was synthesized through epoxidation of tribenzyl glycal **3**, and following by opening of epoxide with ethylthiol. The resulting glucoside **4** was epimerized into mannoside through Swern oxidation and following reduction with $NaBH_4$. The bulky silyl function was introduced at C-2 in **5** in order to enhance the α-mannosylation selectivity.

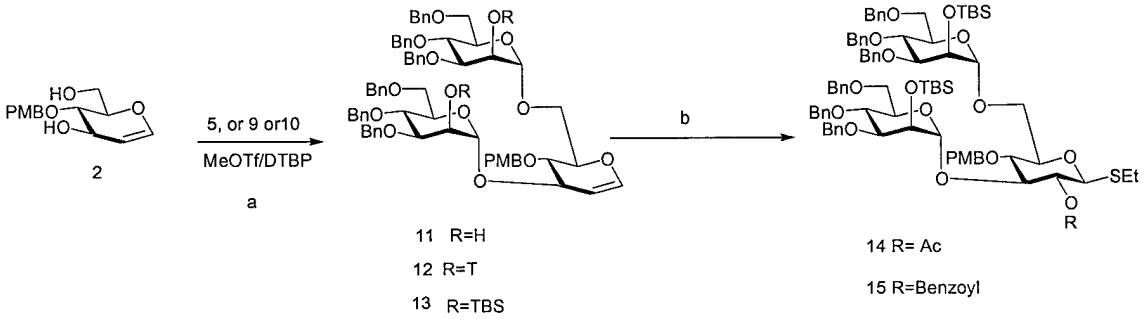


Figure 2 a . MeOTf/DTBP; b. 1. DMDO; 2. HSEt/TFAA; 3. Ac_2O/Py/DMAP or Benzoyl chloride, Et_3N

For a large scale synthesis, the analogous **10**, the α-anomer of mannoside **5**, was prepared through the readily opening of mannose orthoester **6** in presence of $HgBr_2$/EtSH in acetonitrile, leading to α-ethylthiomannoside **7**. After removal of O-acetate, acylation with methylbenzoyl chloride or silylation gave rise to **9** and **10**.

The following coupling between acceptor **2** and **5** , **9** ,or **10** gave rise to trisaccharide **12**, **13**. Using **5** and **10** as donors for coupling, same range of yield (50%) and stereoselectivity was obtained. Better yield (75%) was obtained when **9** was applied. However, the extra step of removing benzoyl ester and then silylation in trisaccharide

stage sacrificed the overall yield. After the large amount of **13** was prepared, the trisaccharide glucal **13** was epoxidated with DMDO under strict anhydrous condition followed by the opening of ethanthiol, catalysized by TFAA. The sugar alcohol can be acylated with acetic anhydride or benzoyl chloride to provide donor **14** and **15** which are armed with acetyl or benzoyl for neighboring participating for β-glycoside formation.

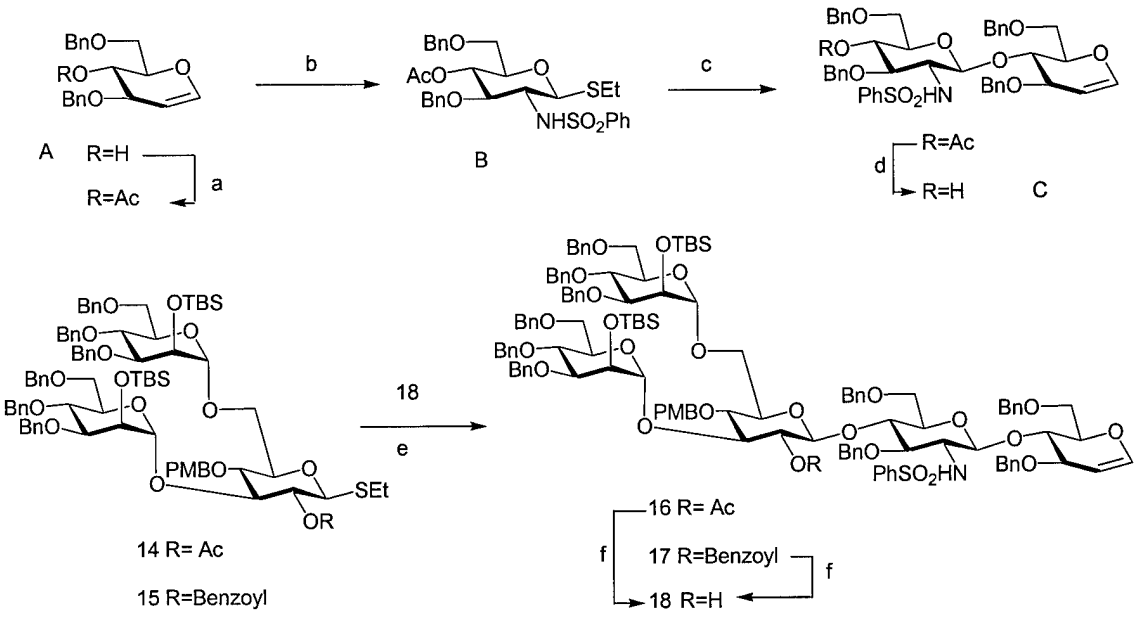


Figure 3. a, Ac_2O/Py/DMAP, 95%; b. 1. IDCP/$PhSO_2NH_2$; 2. EtSH/LiHMDS, 60%; c. A/MeOTf/DTBP/DCM, 65%; d, NaOMe/MeOH; e. MeOTf/TDBP/DCM/**14** , 45% or MeOTf/TDBP/DCM/**15** , 75%, f. **16** or **17** $LiAlH_4$/THF, 98%

The glycosidations between known disaccharide **C** and donor **14** or **15** (Figure 3) were performed in DCM in presence of methyl triflate and DTBP. **16** was obtained in 45% yield with the significant orthoester byproduct formation when **14** was used. Instead, when using benzoylated **15**, the coupling was going very well. The pentasaccharide glycal **17** was obtained in 75%. The acyl function group in compounds **16** and **17** can be readily removed by $LiAlH_4$. The sugar alcohol **18** was epimerized with Dess-Martin oxidation (gave ketone intermediate **19**) and stereoselective reduction with L-seletride to afford trimannose-containing pentasaccharide glycal **20**. Further acetylation gave **21**. It

should be noted the nitrogen in sulfonylamide was also acetylated. With careful control of reaction conditions, those sequences of reactions gave extremely high yield. (82% in four steps from **16** or **17** to **21**). Eventually, chromatographic purification in the four steps can be avoided.

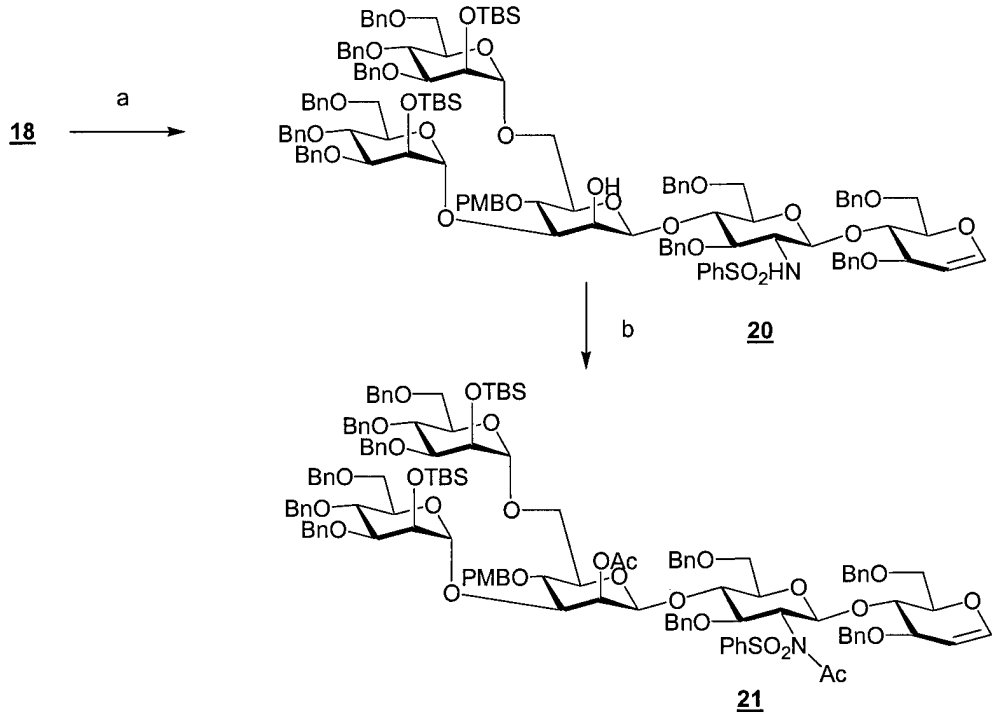


Figure 4. a, 1.\Dess-Martin/Py/DCM/RT; 2. L-seletride/-40°C to r; b. Ac_2O/Py/DMAP, 82% in four steps from **18**

2. Establishment of synthetic methodology of N-linked glycopeptide from glycal

Only a limitted methods to construct N-linked glycopeptide from glycan are available before we worked on this project. And those methods are only applicable to preparation of simple glycopeptide. For sophisticated N-linked glycopeptide, a practical and novel approach needs to be developed. Most ideally, we like to set C—S bond at anomeric position which can survive global deblocking in the sugar body , and then can be converted at later stage into free sugar. Starting from **21**(Figure 5), we did iodosulfomidation followed by rollover reaction with paramethylbenzylthiol to give **22** in

85%. However, the C-S bond(adjacent to benzyl) could not stand the condition(Na/NH_3, Birch conditions) on which we have to rely for global deblocking. This problem was solved by using ethanthiol instead of thiophenol. Thus generated ethylthioglycoside **23** was subjected to removal of TBS with TBAF and then fully deblocking of benznesulfonyl and benzyl. After complete acetylation, all of the O-acetates can be selectively removed in NaOMe/MeOH to afford thioglycoside **27**.

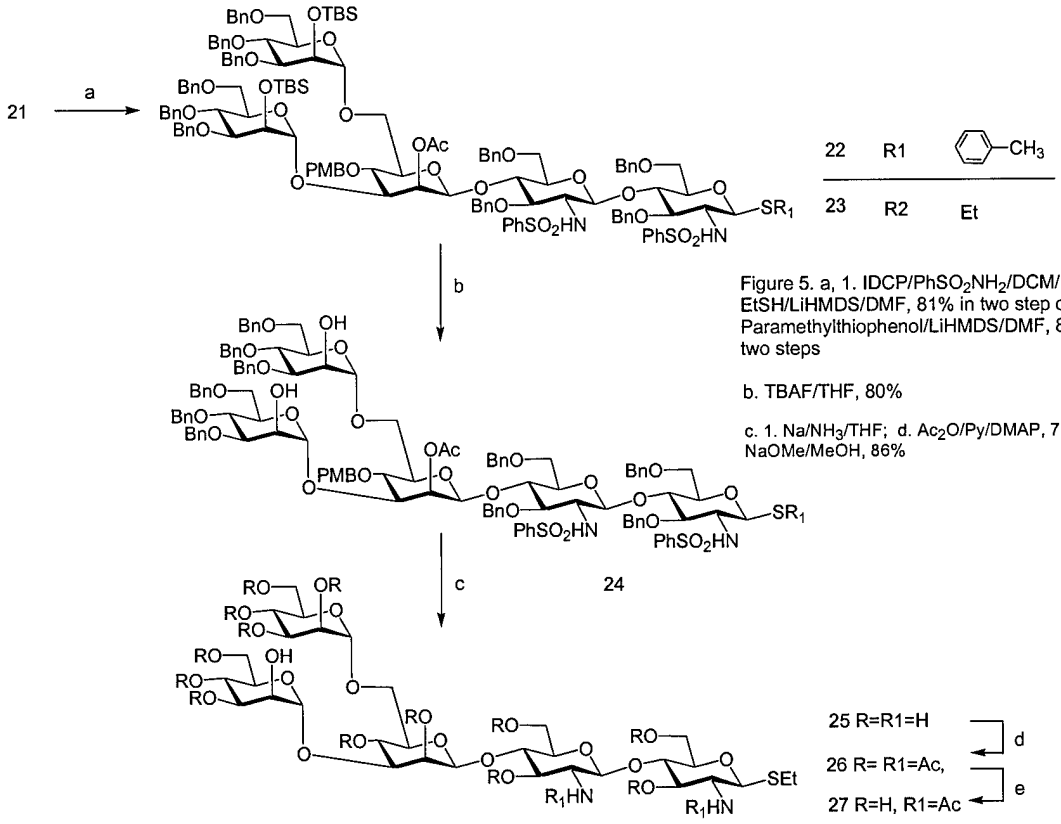


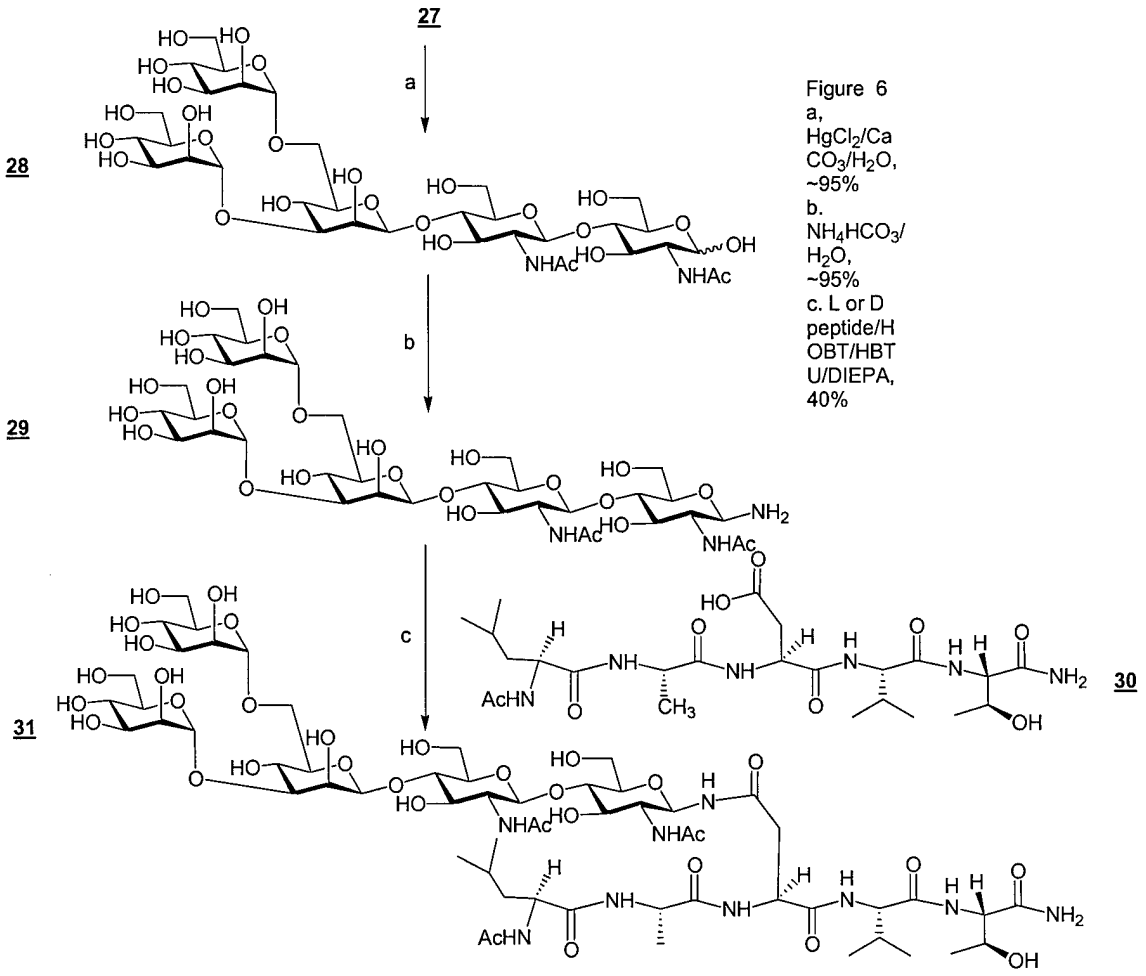
Figure 5. a, 1. IDCP/$PhSO_2NH_2$/DCM/MS4A; 2. EtSH/LiHMDS/DMF, 81% in two step or Paramethylthiophenol/LiHMDS/DMF, 85% in two steps

b. TBAF/THF, 80%

c. 1. Na/NH_3/THF; d. Ac_2O/Py/DMAP, 77%; e, NaOMe/MeOH, 86%

After many experiments, we found excellent conditions in which we were able to convert protecting-free thioglycoside **27** into hemiacetal. Thus, by the agency of $HgCl_2/CaCO_3$ in excess of water, the free pentasaccharide **28** can be obtained in quantitative yield. No any intramolecular cyclization or intermolecular condensation product were detected. By

treatment with excess NH_4HCO_3 in H_2O, **28** can be transformed smoothly into β-glycosylamine. No corresponding α-anomer was found in this reaction.



Pentapeptide **30** containing a free carboxylic acid(from asparagines) was synthesized by automatic synthesizer. HOBT/HBTU promoted coupling of glycosylamine **29** with pentapeptide **30** gave chemical specifically coupling product(Figure 6). Thus the desired β N-linked glycopeptide **31** was obtained in 40% yield. The β-linkage was confirmed by the typical coupling constant J1,2 9.6 Hz at δ4.9 PPM. And no α-isomer was found in

this reaction. Thus we have established a novel methodology to construct N-linked glycopeptide from glycan.

3. Synthesis of specifically designed lactosamine spacer

After establishment of synthetic methodology of β N-Linked glycopeptide, our attention is directed to how to construct the sugar chains. After constant failure in making 15 sugars in our original 5+5+5 strategy, we redesigned our synthesis based on 5+2+3 strategy. For that, a properly protected and reasonably large scale of synthesis of lactosamine derivatives had to be developed. Here described is the synthesis of lactosamine spacer **36**. (Figure 7)

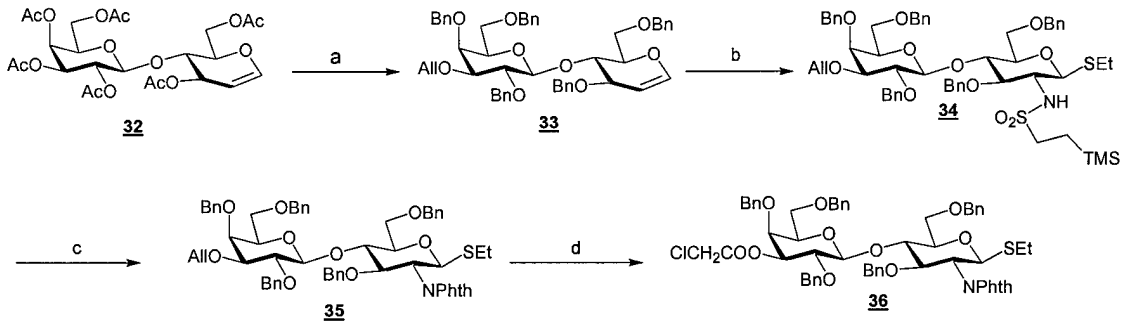


Figure 7. Lactosamine Building Block. a, 1. NH_3/MeOH; 2. Bu_2SnO/benzene; 3. Allyl bromide/Bu_4NBr; 4. BnBr/NaH/DMF, 50%; b, 1. IDCP/$TMSCH_2CH_2SO_2NH_2$, 83%; 2. EtSH/LiHMDS/DMF, 85%; c, 1. CsF/DMF, 90°C, 94%; 2. Phthalic anhydride/Py/rt, 3. Piv_2O or Ac_2O, 100%; d, 1. Ph_3RuCl/DABCO/THF; 1N HCl, 74%; 2. $(ClCH_2CO)_2O$/DTBP/Cat. DMAP/DCM, 97.3%

After deacetylation of **32**, the free lactal was subjected to selective allyl protection of Gal-3 position by the agency of dibutyltin Oxide/allyl bromide at the reflex temperature using benzene as solvent. The rest of hydroxyls were subjected to benzylation, leading to lactal **33**. After standard two-step procedures (1. IDCP/$NH_2SOCH_2CH_2TMS$; 2.

EtSH/LiHMDS/DMF), the protected lactal was converted into thioglycoside **34** in 85%. Giving the poor stereoselectivity of glycosylation while SEM($NHSO_2CH_2CH_2TMS$) is at C-2 position, SEM was removed with CsF in DMF at 95^0C to afford the free amine. The amine function was treated with phthalic anhydride. After complete disappearance of free amine, the mixture was treated with acetic anhydride or Piv_2O, which provides **35** in almost quantitative yield. Thus resulting **35**, which contains the well-proved participating functional group phthamide, is ready for glycosylation. Considering the over-all strategy in this project, we decided to remove the allyl function group first to avoid the potential damage of glycal as an acceptor. Several deallyl conditions were tried($PdCl_2$/MeOH, Ir complex type), it turned out that only Winkenson's catalyst Ph_3RuCl/DABCO/THF, (followed by acetic hydrolysis) works for this purpose. The hydroxyl compound was acylated with chloroacetic anhydride by using DTBP as base to afford **36** in almost quantitative yield.

4. Synthesis of blood type of trisaccharide thioglycoside

Synthesis of building blocks **37**, **38**, **39** have been established in this lab. Epoxidation of **37** with DMDO provides the corresponding α-epoxide, which was coupled with acceptor **38** under the promotion of Lewis acid $ZnCl_2$(Figure 8). The resulting disaccharide was glycosylated with L-fucose fluoride under the agency of $SnCl_2$/AgOTf, leading to protected trisaccharide **40**. In the same sequence as described above for preparation of **36**, the thioglyccoside **43** was obtained in 56% overall yield from **40**.

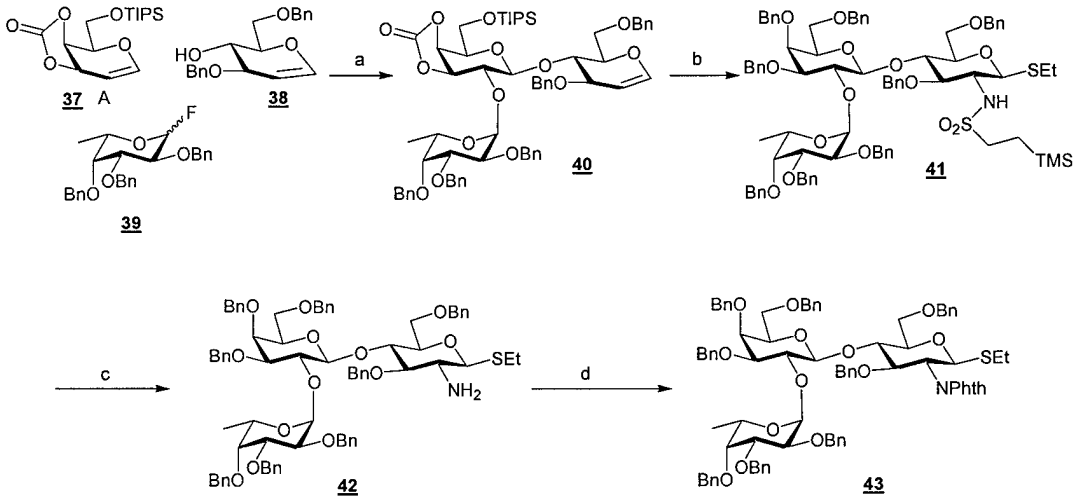


Figure 8. Synthesis of Wing Trisaccharide: a, 1. A/DMDO/DCM; 2. $ZnCl_2$/DCM; 3. $SnCl_2$/AgOTf/DTBP/DCM, 50%; b, 1. TBAF/THF; 2. BnBr/NaH/DMF, 81% in two steps; 3. IDCP/$PhSO_2NH_2$; 4. EtSH/LiHMDS/DMF/-40°C ~0°C, 75% in two steps; c, CsF/DMF/100°C, 5 days 65%; d. 1, Phthalic anhydride; 2. Ac_2O, 97.5%

5. Synthesis of fully Protected "15 mer glycal"

With the large quantity of building block **21**, **36** and **43** at hand, we turned our attention to synthesis of the "15 mer glycal" (Figure 9). At first, the two TBS protecting groups can be removed with TBAF to provide **44**. The following glycosylation with disaccharide **36** promoted by MeOTf/DTBP in ether and

dichloromethane led to nonasaccharide **45**. The two dichloroacetyl groups can be selectively removed using thiourea to give **46** in 99%. The further glycosylation with **43** by the promotion of MeOTf afforded the fully protected **47** in 78%.

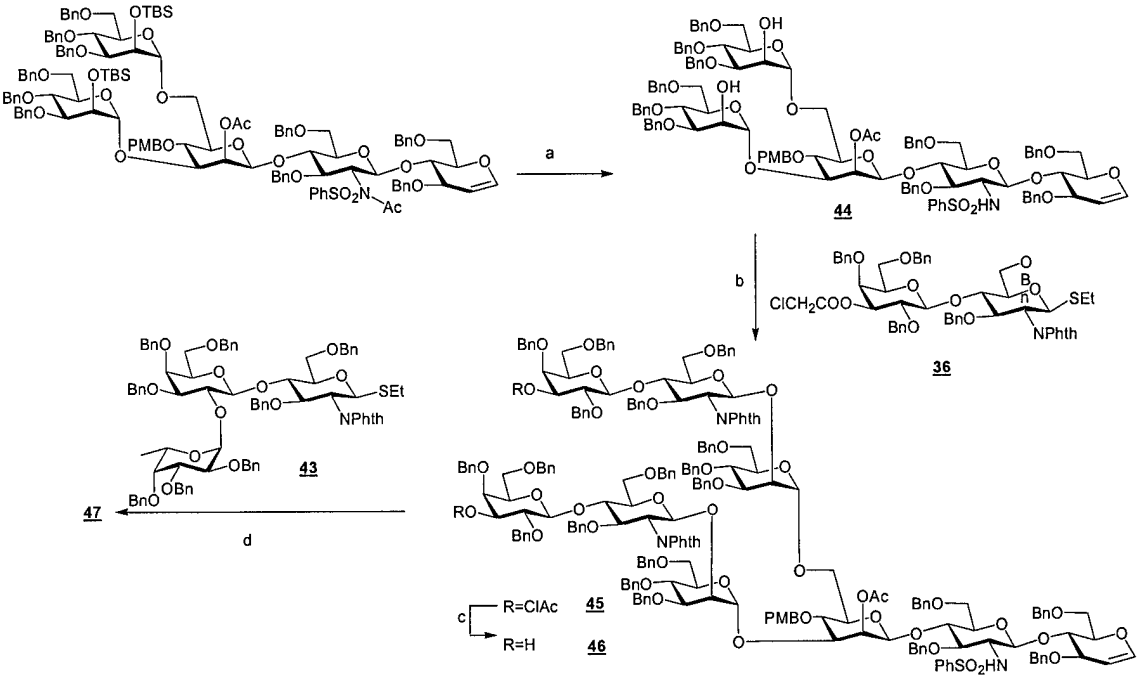
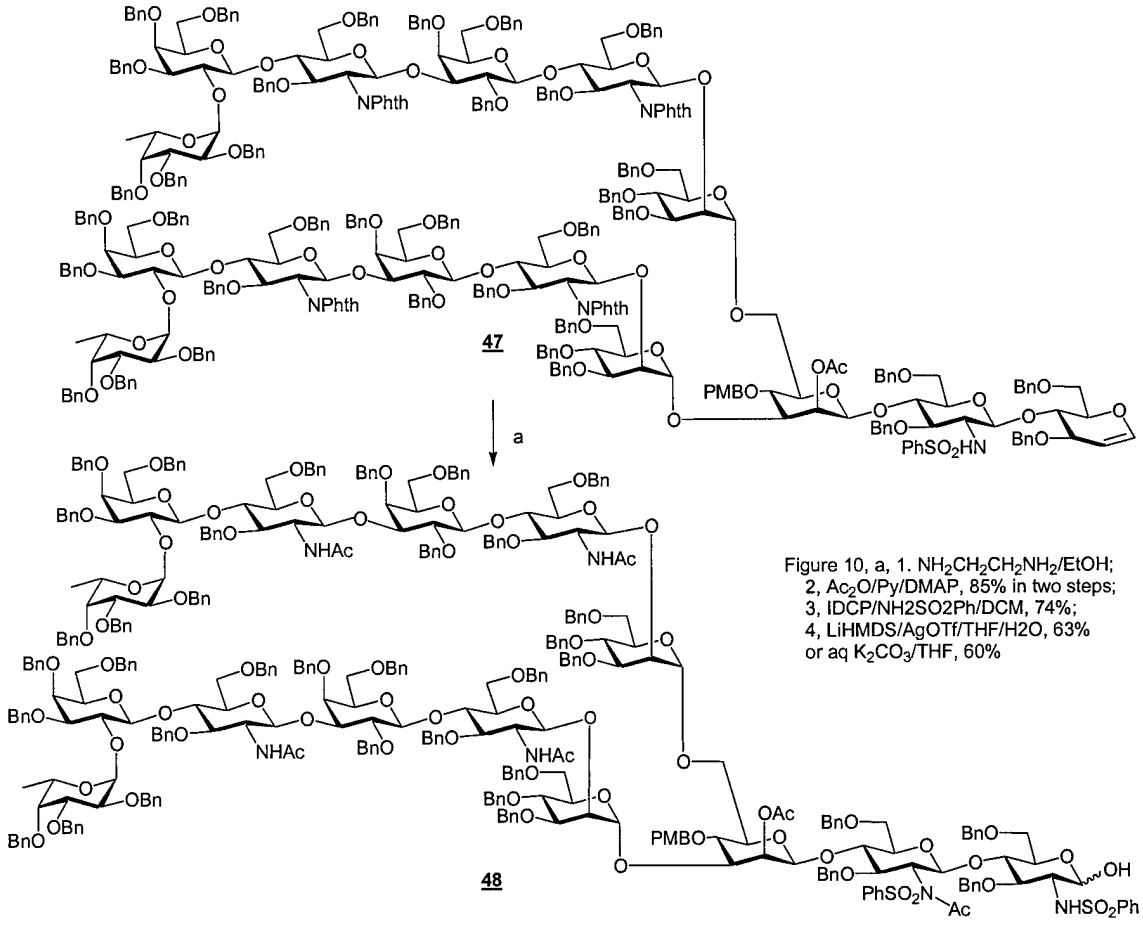


Figure 9. a. TBAF/THF, 77%; b, MeOTf/DTBP/DCM, 0°C~rt, 62%; c, thiourea/$NaHCO_3$/EtOH, 99%; d, MeOTf/DTBP/DCM/ether,78%

6. Successful deprotection of protected glycal **47** and conversion from protected glycal to free hemiacetal **49**

With the successful conversion of pentasaccharide glycal **21** to **23**, we thought it was possible to convert **47** into corresponding thioglycoside based on standard(iodosulphmidation and roll-over) two-step conversion. Unfortunately, the roll-over reaction using thiolate as nuclophile failed. Therefore, we decided to remove the NPhth first with hydrazine, followed by N-acetylation with acetic anhydride. Iodosulphamidation and roll-over using water under both basic and acidic conditions gave rise to hemiacetal **48** in good yield. Once more, we experienced

many difficulties in transforming hemiacetal into ethylthioglycoside, However, we found after several model reactions that sugar aldehyde is remarkably stable to Birch (Na/NH_3) condition while the common aldehyde is very sensitive to that. Therefore, protected hemiacetal **48** can be efficiently converted to free sugar **49** through Birch reduction and N-acetylation (Figure 10).



7. End of Game, the full total synthesis of N-linked glycopeptide

As predicted, direct amination from hemiacetal **49** was conducted in the presence of saturated aq. NH_4HCO_3.(Figure 11). Freeze drying of the reaction mixture gave rise to

glycosylamine **50**. Under the same condition as for **31** using HOBT and HBTU as coupling reagents, peptide **30,** prepared on solid phase, was coupled to **50**. After HPLC purification, the desired glycopeptide **51** was obtained in 20% yield.

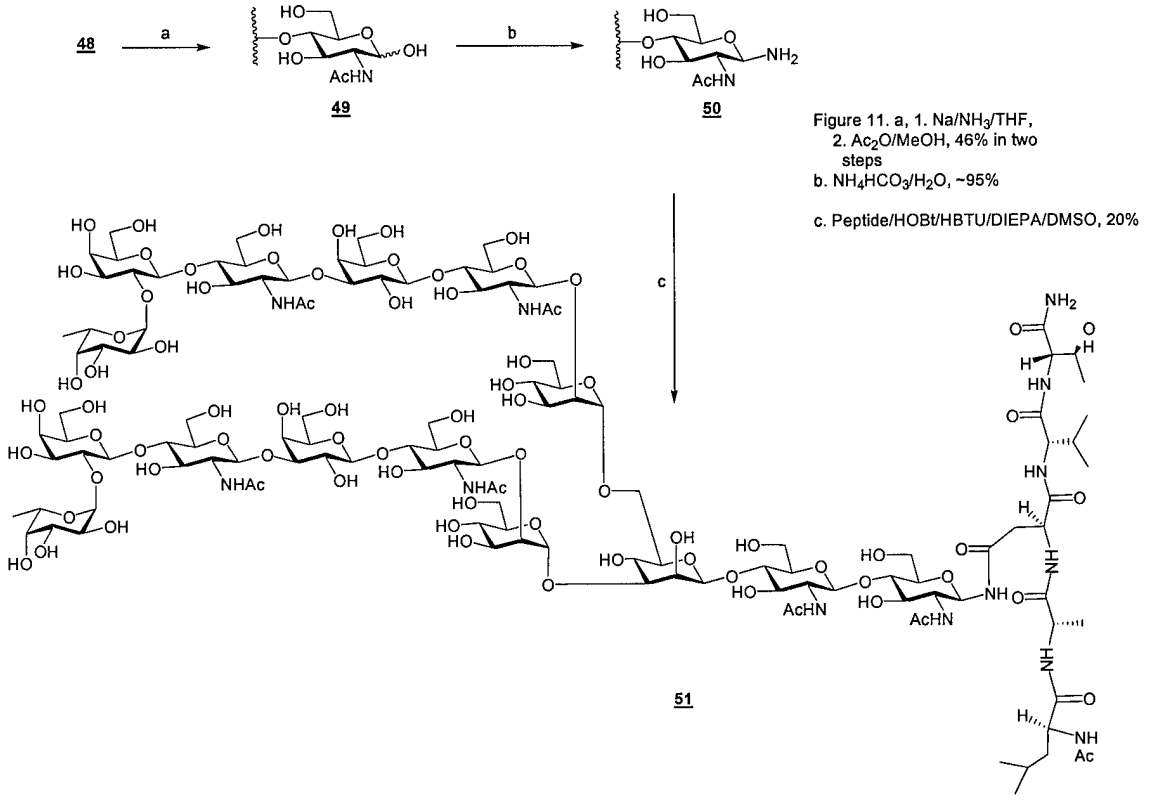


Figure 11. a, 1. $Na/NH_3/THF$, 2. $Ac_2O/MeOH$, 46% in two steps
b. NH_4HCO_3/H_2O, ~95%
c. Peptide/HOBt/HBTU/DIEPA/DMSO, 20%

Key accomplishments:

1. Achievement of the first total synthesis of high mannose N-linked glycopeptide carrying full H-Type II human blood group specificity
2. Development of a large scale synthesis of core mannose structure
3. Development of a novel method to construct N-linked glycopeptide from glycal
4. Development of a practical synthesis of suitably protected lactosamine unit
5. Development of a practical synthesis of H-type of trisaccharide

Reportable Outcomes:

All the above accomplishments are reportable. Some of the results have been published(See enclosed reprints of publication).

Conclusion:

The synthesis described serves to illustrate the powerful application of glycal to the synthesis of biologically important glycoarchetiure. The well development of synthetic method and the extendibility of this chemistry to virtually any type of saccharide presentation will lead to the synthesis of sequence-defined homogenous glycopeptides and thence glycoproteins. This will greatly enhance the biological study on glycoproteins.

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[12] S. Hess, M. Götz, W. B. Davis, M. E. Michel-Beyerle, unpublished results. When intercalated in the DNA duplexes presented in this paper, $\mathbf{X}^+$ displays a rapid excited-state relaxation (on the 50 ps time scale) which leads to energy loss. When a charge-shift reaction can compete with this relaxation, the forward-transfer rate has been shown to be (nearly) free of activation barriers and is faster than the back-transfer rate, which is deep in the Marcus-inverted region of the log (rate) versus energy plot. In this case, the intermediate $\mathbf{X}^\cdot$ is seen in absorption. Conversely, when charge transfer cannot compete with relaxation, there is excited-state energy loss and the activation energy of the forward transfer increases. This leads to kinetics where the rate of charge recombination is faster than that of the forward charge-shift reaction and, thus, the $\mathbf{X}^\cdot$ intermediate is not detectable.

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From Glycals to Glycopeptides: A Convergent and Stereoselective Total Synthesis of a High Mannose N-Linked Glycopeptide**

Zhi-Guang Wang, XuFang Zhang, David Live, and Samuel J. Danishefsky*

Given the elaborate machinery required for the biosynthesis of glycoproteins in cells, it seems likely that such systems perform significant biological functions.[1, 2] Indeed, protein glycosylation has been implicated in mediating protein folding,[3] in protecting against proteolysis,[4] in cellular differentiation,[5] and in cell–cell communication.[6] Major breakthroughs in the detection, purification, sequencing, and spectroscopic analysis of glycans have enabled a growing appreciation of the role of glycobiology in vital life processes.[7] Chemical synthesis[8–10] can play an important role in our understanding of glycobiology by providing access to well-selected, homogeneous, but realistically complex, probe structures for elucidating the relationship of glycoarchitecture and function.[11, 12]

Broadly speaking, glycoproteins are of two major types. In one motif, the terminal galNAc hexose of the saccharide domain is joined to the polypeptide through an α-*O*-glycosidic linkage to the hydroxyl group of a serine (or threonine).[13] The target systems which prompted the research described herein are N-linked glycoproteins, wherein the two domains are joined through a β-N linkage of an asparagine group to a glcNAc unit at the reducing end of the oligosaccharide.[14]

Specifically, we focused on a target where the consensus core high mannose pentamer sequence (see below) would be joined to the peptide domain through a carboxyl group of an Asp side chain (**1**, Scheme 3). Our goals in reaching **1** by chemical synthesis included a concise and efficient assembly of the required oligosaccharide.[15] Clearly, global deprotection of diversely protected functionalities would eventually be required. To this set of specifications we added another, namely, that the fashioning of the asparagine linkage be conducted in a maximally convergent sense with high stereocontrol by joining a fully mature high mannose saccharide to a fully mature peptide. In this way we hoped to pave the way for

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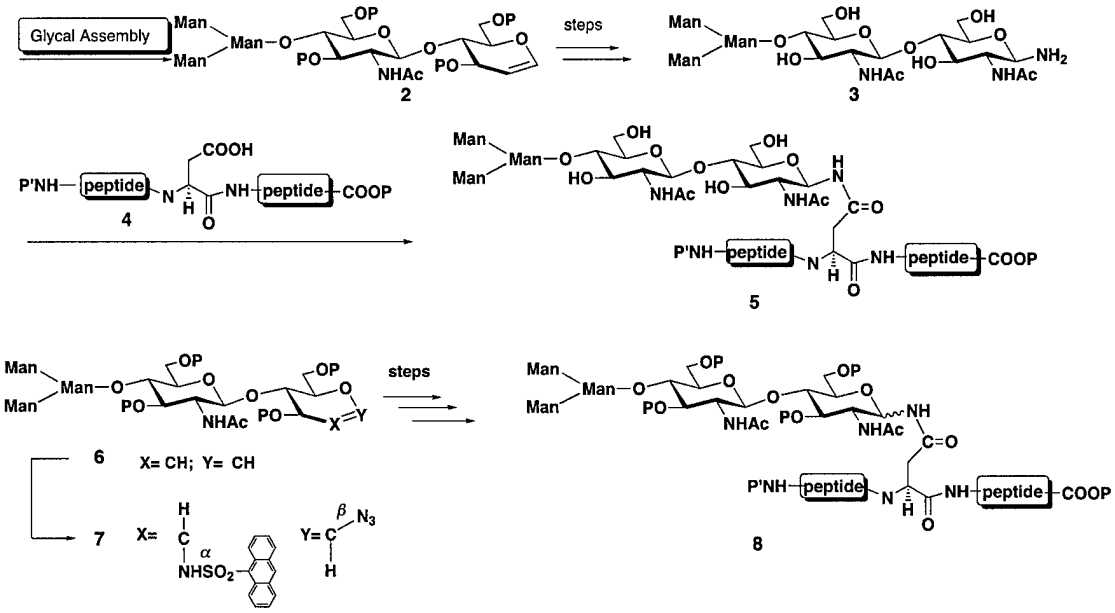
addition of fully synthetic high mannose core structures to preselected aspartate-presenting polypeptides.

Toward this quest, the progression shown in Scheme 1 presented itself. The pertinent high mannose core oligosaccharide would be synthesized as a terminal glycal, thereby taking advantage of the economies of glycal assembly.[16] The double bond would be transformed to the signature chitobiose subunit bearing the β-anomeric amine (**3**). The fully synthetically derived pentamer ensemble would be joined, in a maximally convergent acylation reaction, to the aspartate-presenting peptide, which contains an interior Asp residue and is equipped with a differentiated ω-carboxyl group (**4**). The two central challenges we faced were the conversion of **2**→**3** and the acylation of the latter with **4**, all with tight stereochemical control, en route to **5**.

In a previously reported first generation protocol[17] a fully protected glycal was converted after a complex sequence into a terminal 1-β-azido glcNAc residue (**6** and **7**). However, following reduction of the azido linkage and acylation of the resulting amine with an Asp-containing pentapeptide, the product glycopeptide was obtained as an approximate 1:1 mixture of anomers. During the reduction/acylation sequence, the β anomeric stereochemistry of the azide had been badly compromised en route to the glycopeptide (**8**). A productive solution to the problem of convergence and high stereochemical maintenance in a total synthesis setting is described herein.

As a model for what had to be accomplished, we started with the simple differentiated glycal **9** (Scheme 2). Following implementation of a standard iodosulfonimidation/ethane thiolate rearrangement sequence,[18] **10** was in hand. Conversion of **10**→**11** was conducted as shown. The 2-α-amino group of the latter was selectively acetylated with acetic anhydride in methanol, containing no added base or acylation catalyst, to give **12**. A key step in the model series involved oxidative hydrolylic cleavage of the anomeric β-thioethyl group through the use of *N*-bromosuccinimide (NBS)[19] to produce the model free reducing sugar **13**. Treatment of **13** with ammonium bicarbonate afforded β-anomeric glycosylamine **14**.[20, 21] The latter condensed with Asp-containing peptide **15**, under the conditions indicated, to afford **16**. Remarkably, as was implicit in earlier work of Cohen-Anisfeld and Lansbury,[21, 22] the reaction is highly selective for producing the β-glycosylasparagine-linked glycopeptide. In fact, we did not detect any α-glycoside or aspartoylation through the α-carboxyl group.

The issue to be faced now was whether the methodology used for the simple model could be transferred to a strategically protected high mannose glycal, itself obtained by total synthesis. The logic of the glycal assembly used here had already been charted, but the implementation was now modified and optimized. The "pre-chitobiose glycal" segment **17** was fashioned by the joining of glucal building blocks through azaglycosidation (Scheme 3).[18] A dimannosylated glucosyl donor (**19**) was derived from glycal **18**, itself obtained from the corresponding 4,6-*p*-methoxybenzylidene glycal derivative. Coupling **19** (R = phenyl) with **17** produced the expected β-glucosyl attachment smoothly and stereospecifically. Epimerization at C2[17] (see asterisk) was accomplished by an oxidation/reduction sequence, thereby leading to the protected high mannose glycal (**20**). In the first stage (steps a, b) of converting **20** into **23**, the terminal glycal was used to introduce the 1β-thioethyl (and 2-α-sulfonamido) functions. In step c the silyl protecting groups were removed and in step d both C2 α-amino groups of the sulfonamidochitobiose were exposed. In this case, as opposed to the monosaccharide in Scheme 1, we resorted to peracetylation (all hydroxyl and the two amino groups) for purification. Concurrent deacetylation of all of the ester linkages led to compound **21**. The anomeric thioethyl function suffered cleavage under



Scheme 1. The current and past synthetic strategy from glycal to glycopeptide; P, P': protecting groups.

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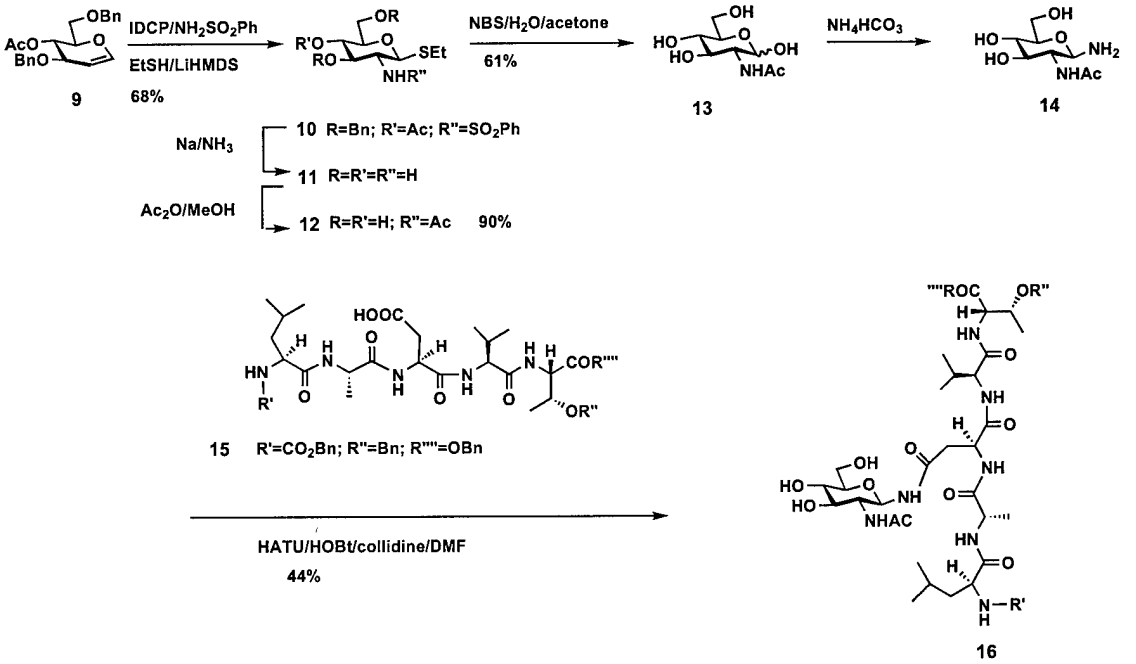
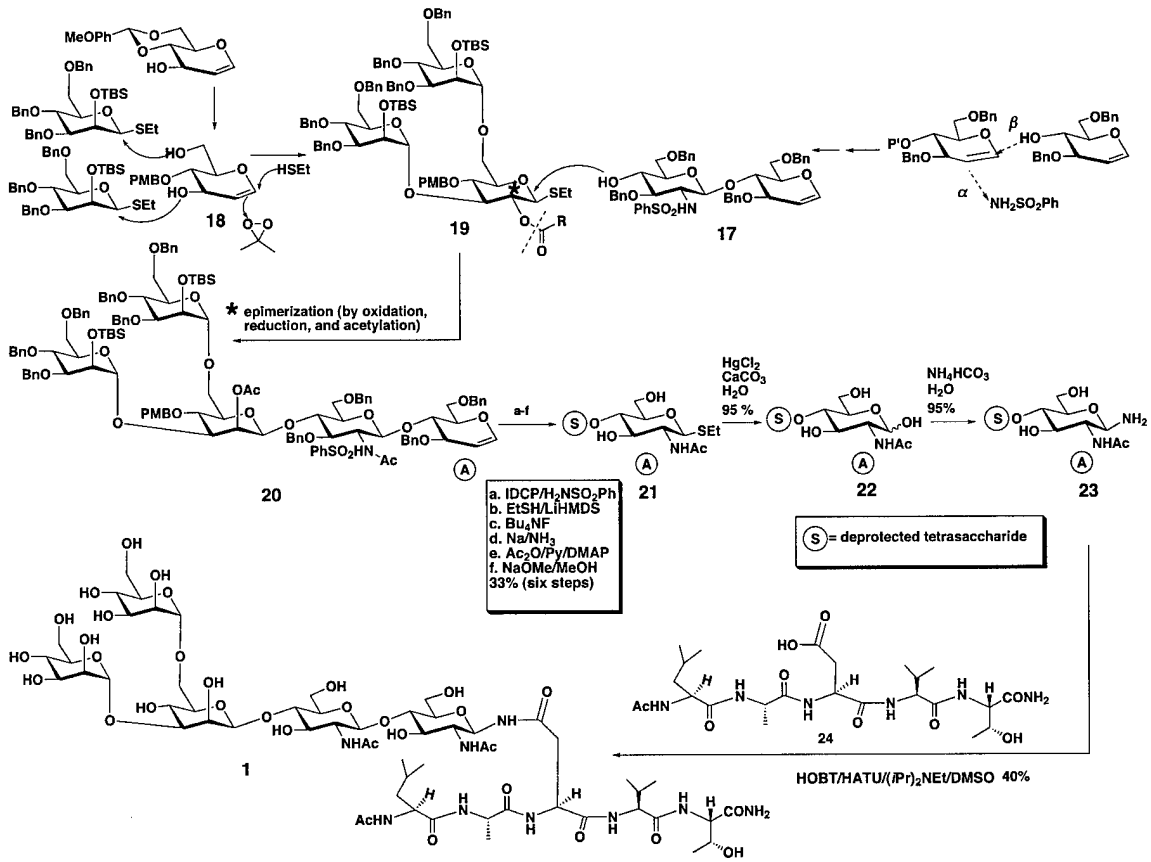


Scheme 2. Bn: benzyl; IDCP: bisdicollidine iodonium perchloride; LiHMDS = lithium hexamethyldisilazanide; HATU: *N*-[(dimethylamino)-1*H*-1,2,3-triazole[4,5-*b*]-pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate; HOBT: 1-hydroxy-1*H*-benzotriazole hydrate; collidine = 2,4,6-trimethylpyridine.



Scheme 3. Synthesis of the high mannose glycopeptide; Py: pyridine; DMAP: 4-dimethylaminopyridine.

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mediation by mercury(II) catalysis (anomers **22**).[23] Once again, aminolysis, following the precedents of Cohen-Anisfeld and Lansbury,[20, 21] led to the pure β-amino anomer **23**. The latter underwent acylation with peptide construct **24**,[24] as shown, to provide the desired homogeneous target **1** (40% after purification by HPLC). The structure assignment of **1** is fully consistent with mass spectral analysis[25] (calcd: *m/z* 1449 [M^+]; found: *m/z* 1472 [M+Na$^+$]).

Furthermore, the ^{1}H NMR spectrum of **1** measured at 800 MHz (Figure 1) is fully supportive of the stereochemical assignment of the five anomeric linkages, including the β-Asn configuration ($\delta = 5.0$, $J = 10$ Hz). The eight NH signals of secondary amides within the glycopeptide were also found and assigned (see inserts). A full assessment of the spectroscopically derived conformation of this highly organized glycopeptide will be published separately.

With proof of principle demonstrated, focus is already directed to new avenues. These include building longer peptide constructs so that the effects of glycosylation on conformation can be probed in detail along a peptide chain. Also underway are experiments where the oligosaccharides entering into the glycopolypeptides present determinants of established biological function. The concise, *totally synthetic* routes to homogeneous glycopeptides demonstrated here will prove to be valuable in furthering progress in glycobiology.

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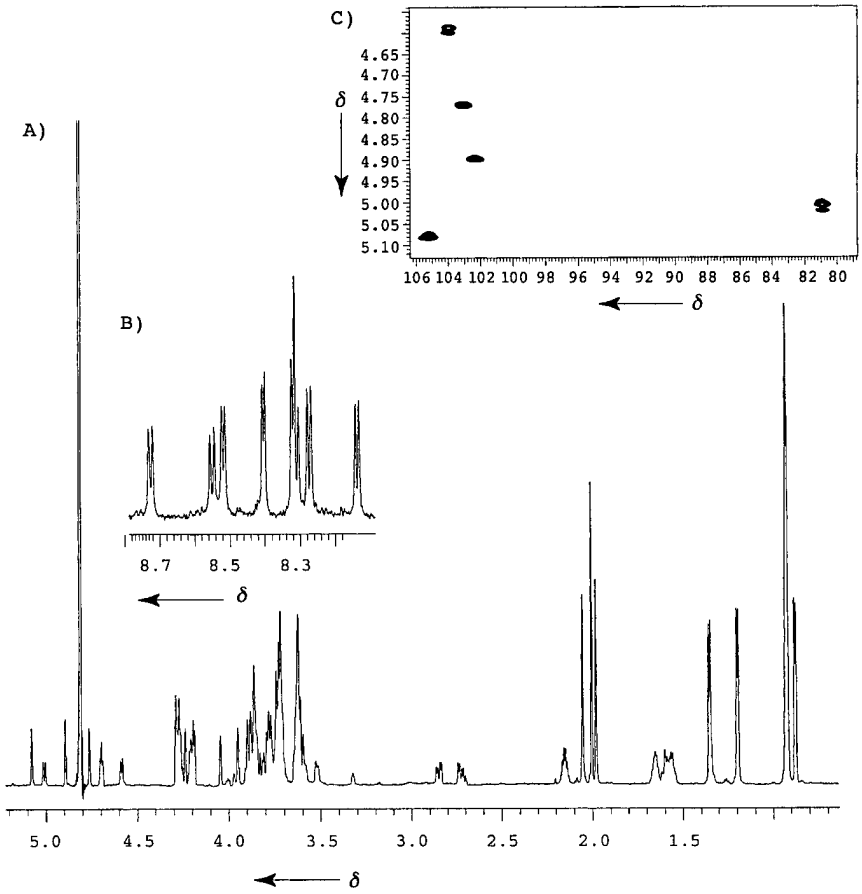

Figure 1. A) ^{1}H NMR (800 MHz) spectrum of **1** in D_2O at 20 °C and pH 3.7 (phosphate buffer). B) Section of the ^{1}H spectrum of **1** in H_2O at 5 °C and pH 3.5 (phosphate buffer) showing the secondary NH signals of amides from the peptide backbone, side chain, and GlcNAc sites. The protons of the terminal carboxamide (not shown) are observed slightly upfield of this region. C) The anomeric region of the ^{1}H-^{13}C HMQC spectrum at 800 MHz of **1** in D_2O at 20 °C and pH 3.7 (phosphate buffer). The GlcNAc anomeric sites at $\delta = 4.58$ and 5.01(^{1}H) are clearly distinguished by their larger ^{1}H couplings.

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Toward Fully Synthetic Homogeneous Glycoproteins: A High Mannose *N*-Linked Glycopeptide Carrying Full H-Type II Human Blood Group Specificity

Zhi-Guang Wang, Xufang Zhang, Michael Visser, David Live, Andrzej Zatorski, Kenneth O. Lloyd and *Samuel J. Danishefsky

Carbohydrate domains in the context of glycoplipids and glycoproteins carry significant messages. The definition of the full scope and impact of oligosaccharide based bioinformatics, falls within the scope of the rapidly growing field of glycobiology.[1] The sorting out of the diverse effects of glycosylation on phenomena ranging from protein folding[2] to cascades bearing on fertilization,[3] inflammation[4] and metastasis,[5] constitutes a major challenge to glycobiology.[6] Other phenomena that are communicated in the grammar of carbohydrate structure include aberrant glycosylation patterns associated with tumorgenesis as well as blood typing.[7] The most widely known of the carbohydrate-centered serology systems, the ABO classification, is based on structural patterns of cell surface glycoproteins on erythrocytes.[8]

From the perspective of chemistry, one of the issues complicating molecular level understanding of the consequences of glycoarchitecture is the phenomenon of heterogeneity. While the various carbohydrate domains present on a glycoprotein may be isolated and purified, this tends to be feasible only after detachment of the oligosaccharide ensemble from its macromolecular setting. One method for dealing with the issue of the inhomogeneity of

glycoproteins is through synthesis – either chemical, enzymatic or through a combination of both.[9]

A long-term goal of our laboratory has been the development of methodology and strategies which would enable the synthesis of complex oligosaccharides bearing glyco based information in a context that simulates the natural glycoprotein setting. As will be shown below, advances in the field are such that a construction is now possible. Our specific focus in this project was a maximally convergent, stereospecific synthesis of a homogeneous glycopeptide bearing an *N*-linked bidomainal high mannose core, connected through lactosamine spacers, to sectors carrying carbohydrate-encoded information. For this demonstration, we focused on the total synthesis of a glycopeptide construct, presenting human H: Type II specificity in a naturally occurring, glycan-like setting. Herein, we describe a solution to this problem in the context of the synthesis of **1**.

Glycal assembly logic had been used for the large-scale syntheses of **2** and **3**.[10,11] Unfortunately, coupling of the glycal epoxide directly available from **3** with **2** occurs (see asterisk at acceptor site 1) at best, in poor yield. A body of chemistry to deal with just such a contingency had been developed (see **3** → **4** → **5**).[12] System **5** had so been organized, such that a C2 hydroxyl in the C ring (see asterisk) could be exposed from a unique benzoate. Epimerization of the resultant alcohol (via oxidation-reduction)[13] followed by acetylation at C2 and deprotection of the unique ring D and ring E silyl ethers, gave rise to **6** with defined acceptor sites at the two "wing mannose" units (see asterisks in **6**).

Figure 1

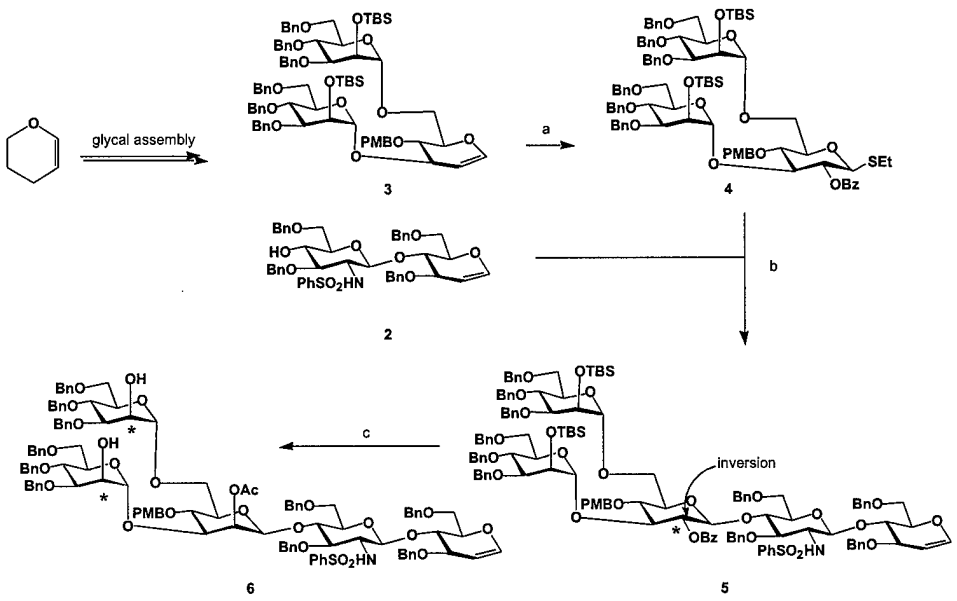

Figure 1. Core Pentasaccharide. a, 1.DMDO/CH_2Cl_2; 2. EtSH/TFAA/CH_2Cl_2, 75% (two steps); 3. BzCl/Py/DMAP, 90% b, MeOTf/ CH_2Cl_2/DTBP,75% ; c, 1. $LiAlH_4$/THF/–20° C- 0° C; 2. Dess-Martin/ CH_2Cl_2/22° C; 3. L-selectride/THF/rt; 4. Ac_2O/Py/DMAP/ CH_2Cl_2, 82% (four steps); 5. TBAF/THF, 77%

We next concerned ourselves with fashioning of the lactosamine spacer region, which characteristically intervenes between the bifurcated high mannose core sector and the bioinformation domains. For purposes of this work, we focused on interpolation of an acetylactosamine element on each wing of the high mannose. However, the chemistry described here could be extended to the building of longer spacer sectors by controlled homocoupling of the bi-directional mannose building block, **11**. Herein, we use **11** as a single insertion unit in the two branching domains.

Our synthesis commenced with per-acetylated lactal which was converted to **8**, bearing a unique allyl protecting group at C3'.[14] Following the chemistry that we had developed with this type of application on the horizon, *trans* diaxial addition of iodonium SES sulfonamide to

the glycal double bond was followed by thiolysis, triggering migration of the sulfonamide from C1 → C2, concurrently with ejection of iodide from C2.[15] Deprotection of the nitrogen and phthaloylation[16] (see **10**) followed by deallylation and reprotection at C3 afforded **11**, equipped with activatable donor and acceptor sites (see asterisks).

Figure 2

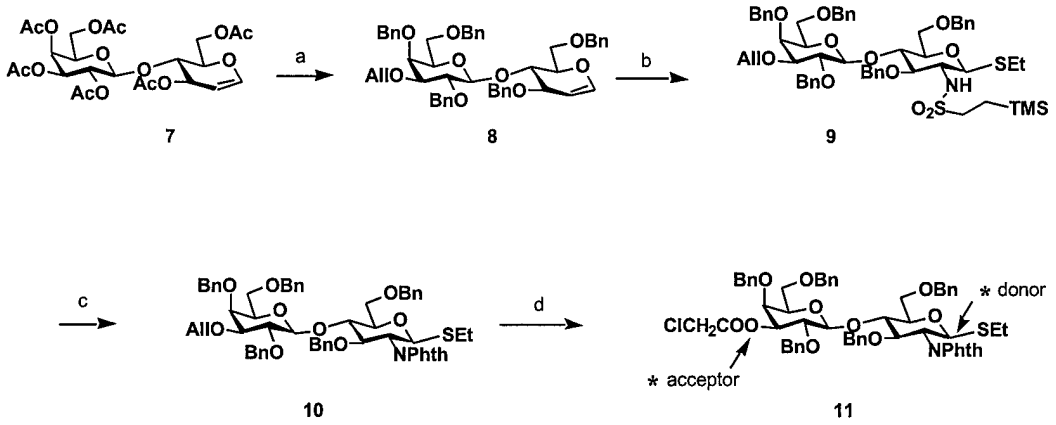

Figure 2. Lactosamine Building Block. a, 1. NH_3/MeOH; 2. Bu_2SnO/C_6H_6; 3. Allyl bromide/Bu_4NBr; 4. BnBr/NaH/DMF 50%; b. 1. IDCP/$TMSCH_2CH_2SO_2NH_2$, 83%; 2. EtSH/LiHMDS/DMF, 85%; c. 1. CsF/DMF, 90° C, 94%; 2. Phthalic anhydride/Py/22° C, 3. Piv_2O or Ac_2O, 100%; d. 1. Ph_3RuCl/DABCO/THF; 1N HCl , 74%; 2. $(ClCH_2CO)_2O$, DMAP/DTBP, 97%

A highly concise fashioning of the H-type antigen is described in Figure 3. The galactal donor system **12** was actuated by strict α-face epoxidation. The α-epoxide, thus produced, coupled to **13** notwithstanding the hindered nature of its acceptor site (see asterisk). The C2' alcohol specifically unveiled in the coupling step, serves as the next acceptor site viz a viz the L-fucosyl donor, **14**. This glycosylation leads to the α-L-fucocide **15**. The glycal found in **15** was advanced to **18** (via **16** and **17**) using chemistry previously developed on simple systems with such applications in mind.

Figure 3

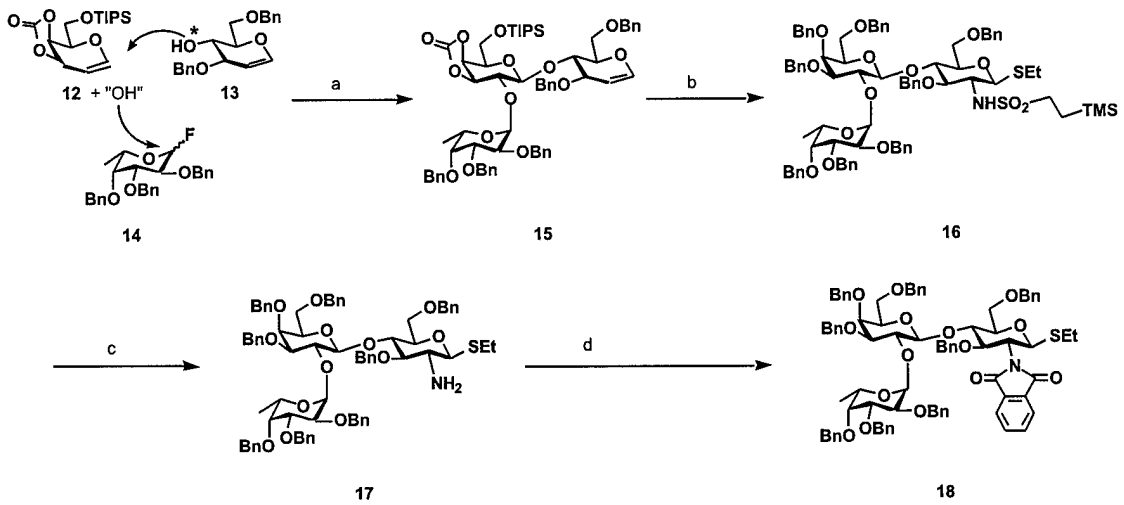

Figure 3. Synthesis of Wing trisaccharide: a, 1.DMDO/CH_2Cl_2; 2. $ZnCl_2$; 3. $SnCl_2$/AgOTf/DTBP/CH_2Cl_2, 50%; b. 1. TBAF/THF; 2. BnBr/NaH/DMF, 81%; 3. IDCP/$PhSO_2NH_2$; 4. EtSLi/DMF/–40° C - 0° C 75%; c. CsF/DMF/100° C, 5 days 65%; d. 1. Phthalic anhydride/Py/22° C; 2. Ac_2O, 97%

We next turned to the assembly of these carefully crafted subunits. The program commenced with two fold coupling of **6** (Figure 1) and **11** (Figure 2). Fortunately, this goal could be accomplished in good yield to produce the 9-mer "**19**" via an apparently stereospecific sulfonamidoglycosylation reaction, modulated by the neighboring phthalamido linkages.[17] The two positionally defined acceptor sites of the nonasaccharide were unveiled as shown (see asterisks in structure **20**). Fortunately, coupling with H-type donor **18** occurs at each of the lactosamine moieties, again under mediation by methyl triflate, to afford 15-mer glycal **21**.

Figure 4

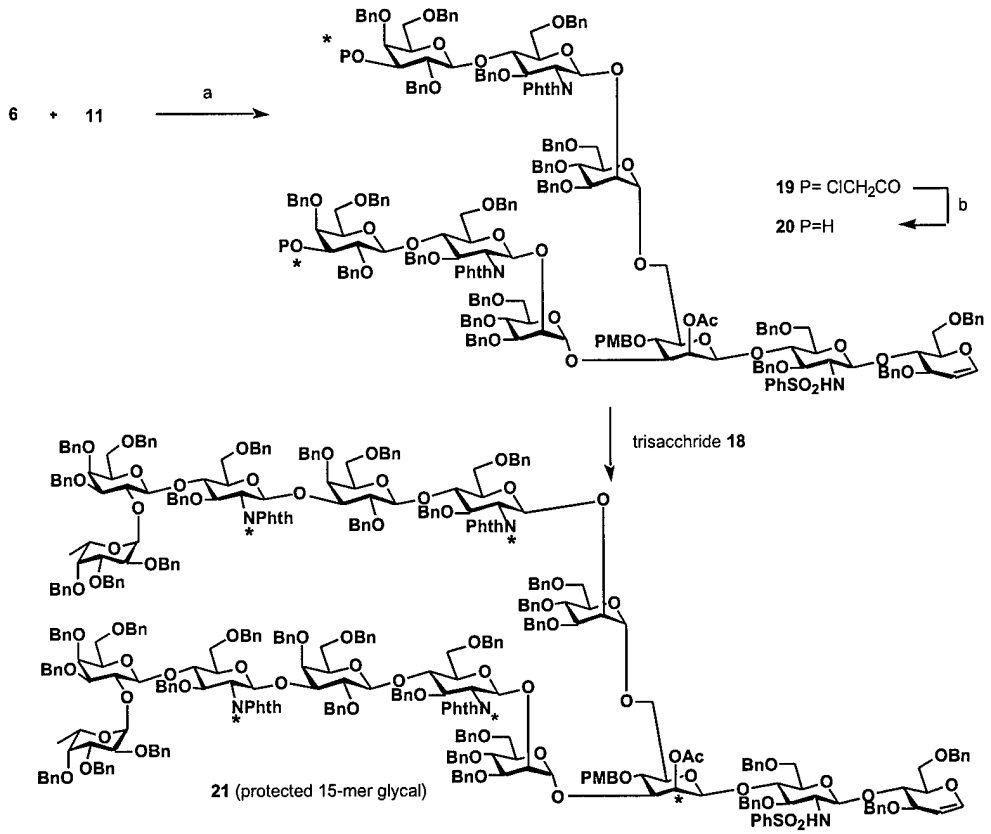

Figure 4. a. MeOTf/ CH_2Cl_2/Et_2O/DTBP, 62%; b. $(NH_2)_2C=S$/EtOH/$NaHCO_3$, 99%, c. trisaccharide **18** / MeOTf/ CH_2Cl_2/Et_2O/DTBP, 78%

Much planning and trial and error research went into accomplishing the global deprotection of the 15-mer. The four phthaloyl groups of **21**, as well as the hindered acetate on the C-ring, were deprotected concurrently. We next carried out the iodosulfonamidation of the glycal linkage at the reducing end of the 15-mer. This addition was followed by hydrolytic rearrangement (a four step sequence, see Figure 5). The critical massive deprotection step could now be realized. *Thus, 37 benzyl groups and the sulfonamido function were all reductively cleaved through the agency of sodium-ammonia (see figure 5, stage A, step 5).*

Remarkably, the terminal hemiacetal linkage was sufficiently robust such that there was no detectable reduction to an alditol in the Birch type deprotection. Furthermore, selective acetylation of the lone amino function in the A ring was readily achieved in the polyhydroxyl substrate system. This remarkable sequence led to the free hemiacetal **22**, which was, in turn, converted to **23** by ammonolysis of the masked aldehyde.[18] In the concluding step, the polyhydroxyl glycosylamine **23** coupled with differentiated Asn pentapeptide **24** to produce desired β-glycosyl N-linked glycopeptide target system **1** (m/e calc'd 3305; found 3328: M + Na). While the yield of this compound may appear modest, detailed NMR analysis fully corroborates that compound **1** meets the standard of homogeneity while displaying its H-type II serological determinant in the context of a high mannose biantennary structure with *N*-acetyllactosamine-like spacer regions. The availability of such an advanced *N*-linked glycopeptide provides excellent opportunities for probing glycoarchitecture and its biological consequences. As seen in figure 6, the proton nmr spectrum of compound **1** at 800 MHz, the nmr spectrum of the 15 mer glyco system linked to the 5-mer peptide is extremely well resolved and suggestive of structural order.

In addition to the critical magnetic resonance and mass spectrometric measurements which support our structural claim on behalf of compound **1**, it was of interest to establish that the H type constructs, so presented, maintain functionality. Indeed, the presence of operational H type II blood group determinants in the synthesized product was confirmed by demonstrating its reactivity with a specific anti-H type II antibody in an ELISA assay[19,20] (Figure 7). Detailed analysis of mutual conformational effects between the oligosaccharide and peptide domains using high level of NMR techniques as well as binding of systems of the type **1** to antibodies of blood ABO determinants will be reported separately.

This synthesis serves to illustrate the current reach of the glycal assembly method. More broadly it speaks to the extraordinary progress fieldwide in oligosaccharide and glycopeptide synthesis. Given the extendibility of this chemistry to virtually any type of saccharide presentation, and the capacity, in principle, for ligation of the pentapeptide construct to larger polypeptide or protein domains,[21] the synthesis of sequence-defined homogeneous glycopeptides and thence glycoproteins is at hand. Studies directed toward reaching fully functional glycoproteins by total synthesis are underway.

Figure 5

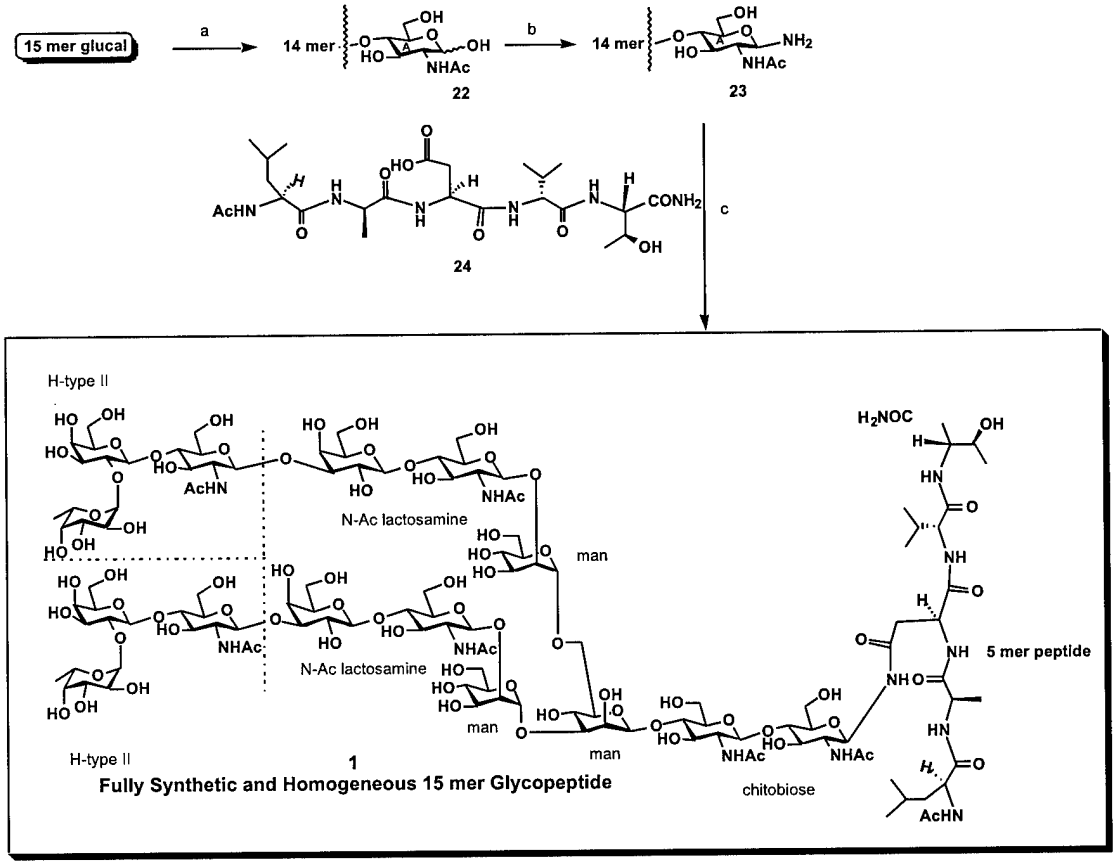

Figure 5. a.1. $NH_2CH_2CH_2NH_2$/EtOH; 2. Ac_2O/Py/DMAP, 85% (two steps); 3. IDCP/NH_2SO_2Ph, 74%; 4. LiHMDS/AgOTf/THF/H_2O,63%; 5. Na/NH_3/THF; 6. Ac_2O/MeOH, 46% (two steps); b. NH_4HCO_3/H_2O, 90%; c. Peptide **24**/HOBT/HBTU/DIEPA/DMSO, 20%

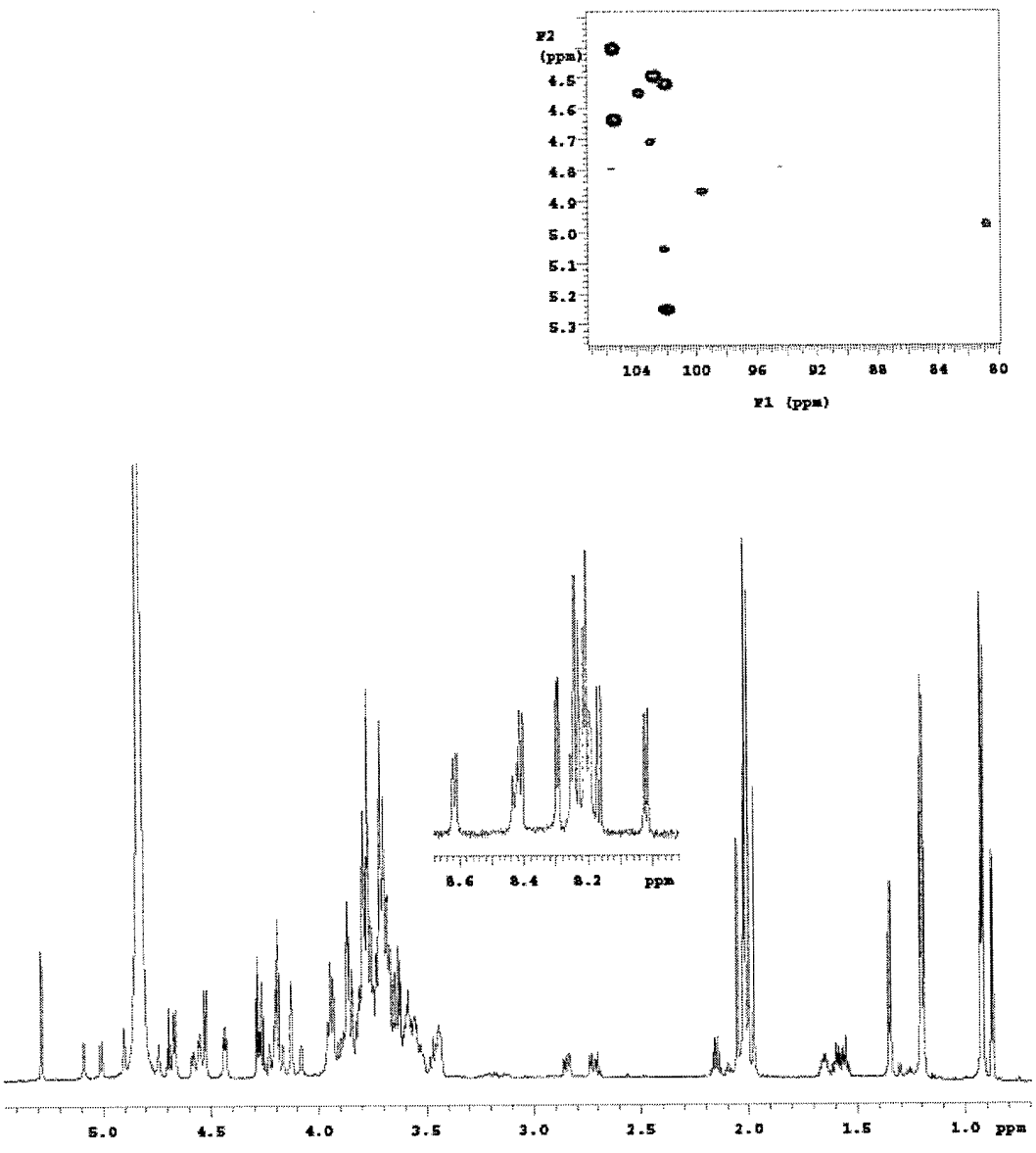

Figure 6. Proton NMR Spectrum of Compound **1**

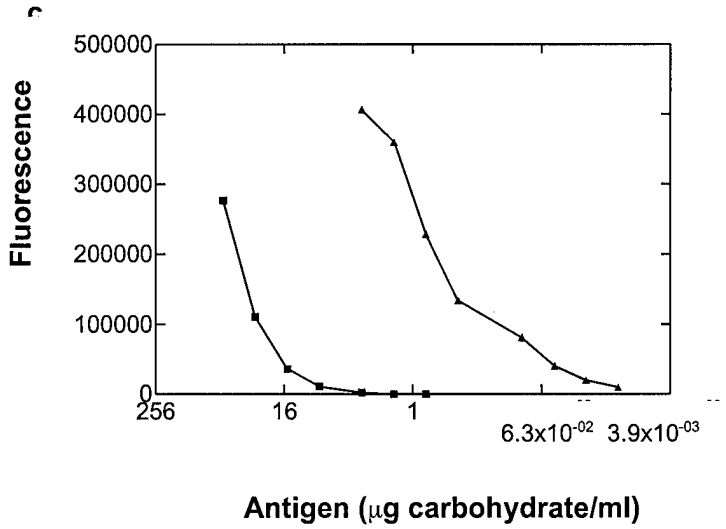

Figure 7. Reactivity of glycopeptide (■) and HII-active mucin (▲) with anti-H type II antibody (mAB; See ref. 18) as determined by ELISA.

Keywords: glycals, glycopeptides, gloycosylamines, glycosylations, total synthesis

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Figure Captions:

Figure 1. Core Pentasaccharide. a, 1.DMDO/CH_2Cl_2; 2. EtSH/TFAA/CH_2Cl_2, 75% (two steps); 3. BzCl/Py/DMAP, 90% b, MeOTf/ CH_2Cl_2/DTBP,75% ; c, 1. $LiAlH_4$/THF/–20° C- 0° C; 2. Dess-Martin/ CH_2Cl_2/22° C; 3. L-selectride/THF/rt; 4. Ac_2O/Py/DMAP/ CH_2Cl_2, 82% (four steps); 5. TBAF/THF, 77%

Figure 2. Lactosamine Building Block. a, 1. NH_3/MeOH; 2. Bu_2SnO/C_6H_6; 3. Allyl bromide/Bu_4NBr; 4. BnBr/NaH/DMF 50%; b. 1. IDCP/$TMSCH_2CH_2SO_2NH_2$, 83%; 2. EtSH/LiHMDS/DMF, 85%; c. 1. CsF/DMF, 90° C, 94%; 2. Phthalic anhydride/Py/22° C, 3. Piv_2O or Ac_2O, 100%; d. 1. Ph_3RuCl/DABCO/THF; 1N HCl , 74%; 2. $(ClCH_2CO)_2O$, DMAP/DTBP, 97%

Figure 3. Synthesis of Wing trisaccharide: a, 1.DMDO/CH_2Cl_2; 2. $ZnCl_2$; 3. $SnCl_2$/AgOTf/DTBP/CH_2Cl_2, 50%; b. 1. TBAF/THF; 2. BnBr/NaH/DMF, 81%; 3. IDCP/$PhSO_2NH_2$; 4. EtSLi/DMF/–40° C - 0° C 75%; c. CsF/DMF/100° C, 5 days 65%; d. 1. Phthalic anhydride/Py/22° C; 2. Ac_2O, 97%

Figure 4. a. MeOTf/ CH_2Cl_2/Et_2O/DTBP, 62%; b. $(NH_2)_2C$=S/EtOH/$NaHCO_3$, 99%, c. trisaccharide **18** / MeOTf/ CH_2Cl_2/Et_2O/DTBP, 78%

Figure 5. a.1. $NH_2CH_2CH_2NH_2$/EtOH; 2. Ac_2O/Py/DMAP, 85% (two steps); 3. IDCP/NH_2SO_2Ph, 74%; 4. LiHMDS/AgOTf/THF/H_2O,63%; 5. Na/NH_3/THF; 6. Ac_2O/MeOH, 46% (two steps); b. NH_4HCO_3/H_2O, 90%; c. Peptide **24**/HOBT/HBTU/DIEPA/DMSO, 20%

Figure 6. Proton NMR Spectrum of Compound **1**

Figure 7. Reactivity of glycopeptide (■) and HII-active mucin (▲) with anti-H type II antibody (mAB; See ref. 18) as determined by ELISA.